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Varicella-Zoster Virus ORF12 Protein Activates the Phosphatidylinositol 3-Kinase/Akt Pathway To Regulate Cell Cycle Progression

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Varicella-zoster virus (VZV) activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and alters cell cycle progression, but the viral protein(s) responsible for these activities is unknown. We previously reported that the VZV open reading frame 12 (ORF12) protein triggers phosphorylation of ERK. Here, we demonstrate that the VZV ORF12 protein also activates the PI3K/Akt pathway to regulate cell cycle progression. Transfection of cells with a plasmid expressing the ORF12 protein induced phosphorylation of Akt, which was dependent on PI3K. Infection of cells with wild-type VZV triggered phosphorylation of Akt, while infection with an ORF12 deletion mutant induced less phosphorylated Akt. The activation of Akt by ORF12 protein was associated with its binding to the p85 subunit of PI3K. Infection of cells with wild-type VZV resulted in increased levels of cyclin B1, cyclin D3, and phosphorylated glycogen synthase kinase 3β (GSK-3β), while infection with the ORF12 deletion mutant induced lower levels of these proteins. Wild-type VZV infection reduced the G_1 phase cell population and increased the M phase cell population, while infection with the ORF12 deletion mutant had a reduced effect on the G_1 and M phase populations. Inhibition of Akt activity with LY294002 reduced the G_1 and M phase differences observed in cells infected with wild-type and ORF12 mutant viruses. In conclusion, we have found that the VZV ORF12 protein activates the PI3K/Akt pathway to regulate cell cycle progression. Since VZV replicates in both dividing (e.g., keratinocytes) and nondividing (neurons) cells, the ability of the VZV ORF12 protein to regulate the cell cycle is likely important for VZV replication in various cell types in the body.

The serine-threonine protein kinase B/Akt acts downstream of phosphatidylinositol 3-kinase (PI3K) and functions as an essential signaling molecule for many growth factor-induced responses, including cell cycle regulation, cell metabolism, proliferation, and survival (1). In unstimulated cells, Akt resides within the cytosol in a catalytically inactive state. The activation of PI3K leads to the generation of phosphatidylinositol triphosphate, which then recruits Akt and phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane in order to phosphorylate Akt at threonine 308 by PDK1. The full activation of Akt also requires phosphorylation at serine 473 by other kinases, such as mammalian target of rapamycin complex 2 (mTOR2). Once activated, Akt phosphorylates several downstream targets, including glycogen synthase kinase 3 (GSK-3) (2), cyclin D1, cyclin-dependent kinase inhibitor $p27^{Kip1}$ (3), the proapoptotic Bcl-2 family member Bad (4), and mTOR1 (5) to affect cell proliferation, cell cycle progression, cell survival, and protein synthesis.

Since the PI3K/Akt pathway promotes cell survival, protein synthesis, and cell proliferation, which are beneficial to virus replication, many DNA and RNA viruses activate Akt during lytic infection. These include herpes simplex virus 2 (HSV-2), Epstein-Barr virus (EBV), influenza A, and human T-lymphotropic virus type 1 (HTLV-1). HSV-2 expresses the ribonucleotide reductase (ICP10) to activate Akt (6), block apoptosis (7), and induce viral gene transcription (8). EBV activates PI3K/Akt to inhibit FOXO3, repress DNA repair (9), and promote cell survival (10, 11) and cell transformation (12). Influenza A virus activates PI3K to enhance viral replication (13) and antiapoptotic signaling responses (14). HTLV-1 Tax activates Akt to increase virus replication (15) and cell transformation (16). In contrast, other viruses inhibit the Akt pathway to suppress host immune responses. For example, measles virus inactivates PI3K/Akt to induce T-cell unresponsiveness (17), and vesicular stomatitis virus (VSV) matrix protein inactivates Akt so that it might inhibit the interferon response that could otherwise inhibit virus replication (18).

Virus replication can result in the depletion of cell nutrients, hypoxia, and endoplasmic reticulum stress, which can block transduction of Akt signals, and can also result in the inhibition of cap-dependent translation through the inactivation of mTOR1 activity and activation of apoptotic responses (5). All of these activities have deleterious effects on the replication of DNA viruses. Thus, DNA viruses must be able to not only activate the PI3K/Akt pathway but also counteract the inhibition of this pathway that results from stress signaling (19).

VZV infection activates the PI3K/Akt pathway to promote virus replication, and virus-encoded protein kinases have been shown to be important for the activation of Akt (20). Here, we show that the VZV ORF12 protein activates Akt in a PI3K-dependent manner by its association with p85 and that ORF12 protein activation of PI3K/Akt contributes to cell cycle regulation.

MATERIALS AND METHODS

Cells, viruses, plasmids, and luciferase reporter assays. Human embryonic kidney cells (HEK293T) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), while melanoma (MeWo) and MRC-5 cells were grown in minimum

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essential medium (MEM) containing 10% FBS. VZV infections were performed using cell-associated viruses. The viruses, plasmids, and luciferase reporter assays we used were described previously (21). LY294002 (Cell Signaling Technology) was added to the cells in some experiments to inhibit PI3K activity.

Immunoblotting. Infected cell lysates were fractionated on polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with rabbit anti-VZV IE62 antibody (a gift from Paul Kinchington, University of Pittsburgh), p85/55 (Millipore), rabbit anti-p-ERK1/2, ERK1/2, p-Akt, Akt, p-GSK-3β (S9), GSK-3β, cyclin B1, cyclin D1, cyclin D3, $p27^{Kip1}$, Rictor, p-PTEN, mTOR, p-mTOR(S2448), p85, PI3K III (Cell Signaling Technology), mouse anti-V5 (Serotec), Flag (Sigma), VZV glycoprotein E (gE) (MAB8612; Chemicon), or actin antibody (Sigma).

Cell cycle analysis by propidium iodide and VZV gE staining. MRC-5 cells were plated onto 6-well plates for 48 h until they were confluent and then were infected with MeWo cell-associated virus at a multiplicity of infection (MOI) of 0.1. At 24 h after infection, cells were incubated on ice with 0.5 ml 0.1% trypsin and 2 μM EDTA. When the cells detached from the plates, 3 ml MEM containing 10% fetal bovine serum (FBS) was added, and the cells were pelleted at 311 × *g* for 5 min. The cells were then resuspended in 1 ml cold phosphate-buffered saline (PBS), and the cell suspension was pipetted into 2.5 ml of absolute ethanol and incubated on ice for 15 min to fix the cells. The fixed cells were pelleted at 485 × *g* for 5 min and washed with 3 ml cold PBS, resuspended in 500 μl propidium iodide (PI) staining solution (PBS with 50 μg/ml PI, 0.1 mg/ml RNase A, and 0.05% Triton X-100) containing anti-VZV gE antibody (1:500 dilution) (MAB8612; Chemicon), and incubated for 60 min at 37°C. Three milliliters PBS was added, the cells were pelleted at 485 × *g*, resuspended in 0.5 ml PBS plus 0.05% Triton X-100 (PBST) containing Alexa Fluor 488 goat anti-mouse IgG (1:1,000 dilution) (Invitrogen) for 60 min at 37°C, pelleted, and washed with PBS, and flow cytometry was performed.

Transfection of cells with plasmids. MRC-5 cells were transfected with a plasmid expressing ORF12 tagged with a V5 epitope at the C terminus or pcDNA3.1 (Invitrogen) using Nucleofector Kit R (Lonza, Germany), and 3 days later, the cells were harvested for immunoblotting.

RESULTS

Expression of ORF12 protein activates Akt. Prior studies have shown that VZV infection activates several host signaling molecules, including ERK, p38, JNK, and PI3K/Akt. The VZV ORF61 protein was reported to activate JNK (22), VZV protein kinases ORF47 and ORF66 were shown to activate Akt (20), and we have reported that ORF12 protein activates ERK and p38 (21). Since HSV infection activates PI3K/Akt (6) and HSV-1 VP11/12 is required for Akt activation (23), we hypothesized that the VZV ORF12 protein, the ortholog of HSV VP11/12, might also activate Akt.

To test this hypothesis, we transfected HEK293T cells with plasmids expressing VZV ORF12 protein, VZV ORF13 protein, empty vector (pcDNA3.1), or c-Src (a known activator of Akt). At 48 h after transfection, cell lysates were prepared and assayed for p-Akt, p-ERK1/2, total Akt, total ERK1/2, ORF12 protein, ORF13 protein, and actin (Fig. 1A). HEK293T cells expressed high levels of phosphorylated Akt after transfection with empty vector. Higher levels of p-Akt were observed in cells transfected with ORF12 and c-Src than in cells transfected with empty vector or VZV ORF13 (Fig. 1A). These results indicate that Akt is constitutively activated in HEK293T cells, while the expression of VZV ORF12 further enhances Akt phosphorylation. To verify that ORF12 protein is functional in the cells, we determined whether the protein could phosphorylate ERK. ORF12 and c-Src proteins,

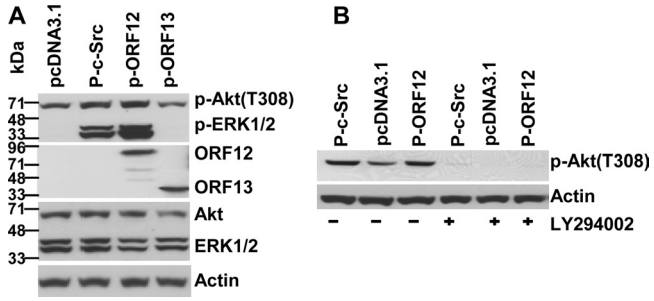

FIG 1 VZV ORF12 protein activates Akt. (A) HEK293T cells were transfected with plasmids expressing the VZV ORF12 or ORF13 protein containing a V5 epitope tag at the C terminus of the protein, c-Src (a known activator of Akt), or pcDNA3.1 (empty vector control). After 48 h, cells lysates were immunoblotted with antibody to p-Akt, p-ERK1/2, V5, Akt, ERK1/2, or actin. Molecular mass markers are shown on the left. (B) MeWo cells were transfected with plasmids expressing c-Src, pcDNA3.1, or the VZV ORF12 protein. Forty-two hours later, cells were treated with LY294002 at 20 μM or DMSO (the solvent for LY294002) for an additional 6 h, and cell lysates were immunoblotted with antibodies to p-Akt (T308) or actin.

but not ORF13 protein, activated ERK, which is consistent with our previous results (21).

To further confirm the ability of ORF12 protein to activate the PI3K/Akt pathway, MeWo cells were transfected with plasmids expressing ORF12 protein, c-Src, or empty vector, and after 48 h, the transfected cells were treated with the PI3K inhibitor LY294002 or dimethyl sulfoxide (DMSO) (the solvent for LY294002) for 6 h. Expression of ORF12 protein or c-Src resulted in increased phosphorylation of Akt compared with empty vector (Fig. 1B). The inhibition of PI3K activity by treatment of the cells with LY294002 reduced phosphorylation of Akt by either ORF12 protein or c-Src to levels similar to those observed with empty vector. Altogether, these results indicate that ORF12 protein activates Akt and that the activation is PI3K dependent.

ORF12 protein triggers phosphorylation of Akt in VZV-infected cells. To determine whether ORF12 protein is responsible for the phosphorylation of Akt in VZV-infected cells, MRC-5 cells were infected with either VZV recombinant Oka (ROka) (parental virus) or ROka12D (VZV deleted for ORF12) (21). While VZV ROka phosphorylated Akt at 12 h after infection, and the phosphorylation increased at 24 and 36 h after infection, cells infected with ROka12D showed less increase in phosphorylated Akt during the same time points (Fig. 2A).

While PI3K is the major kinase responsible for the activation of Akt, a previous report postulated that Akt phosphorylation may be independent of PI3K in VZV-infected MeWo cells (20). Since we found that the expression of ORF12 protein in transfected cells can phosphorylate Akt (Fig. 1A) and that this activation of Akt is PI3K dependent (Fig. 1B), we sought to determine whether the activation of Akt is also PI3K dependent in VZV-infected cells. MRC-5 cells were infected with VZV ROka or ROka12D for 24 h and then were left untreated or were treated with the PI3K inhibitor LY294002 or MEK inhibitor U0126 for 6 h. Akt was phosphorylated in cells infected with VZV ROka, but little phosphorylation was detected in cells infected with ROka12D (Fig. 2B). In contrast, treatment of the cells with the PI3K inhibitor markedly reduced Akt phosphorylation in the cells infected with VZV ROka. To verify that activation of Akt by ORF12 is directly through PI3K and not through ERK (since there may be cross talk

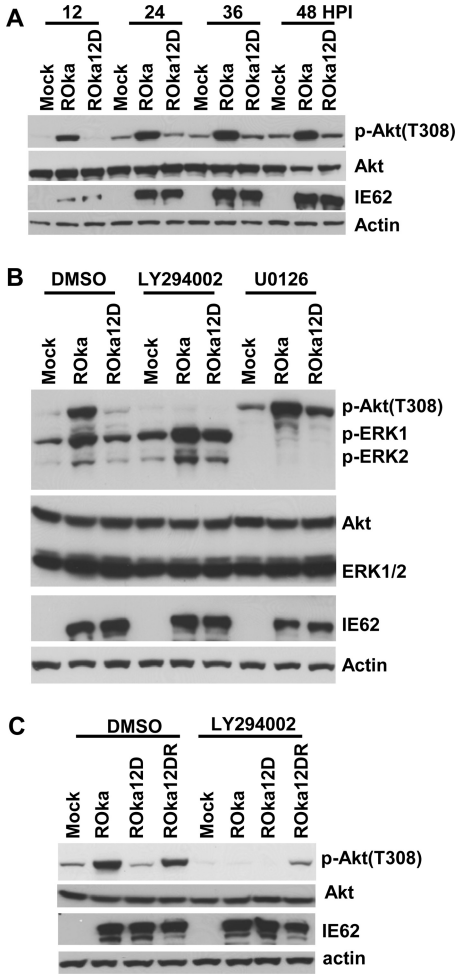

FIG 2 Deletion of ORF12 from VZV reduces phosphorylation of Akt in virus-infected cells. (A) Immunoblots of MRC-5 cells infected for various times with 0.1 PFU/cell of ROka or ROka12D. HPI, hours postinfection. (B and C) MRC-5 cells were infected with 0.1 PFU/cell of ROka, ROka12D, or ROka12DR for 24 h, treated with DMSO, 20 μM LY294002, or 20 μM U0126 for an additional 6 h, and then immunoblotted with antibody to p-Akt, total Akt, p-ERK, total ERK, the VZV IE62 protein, or actin.

between these two signaling pathways), we treated cells with a MEK inhibitor. Inhibition of MEK abolished the activation of ERK by VZV ROka but did not inhibit the activation of Akt by ROka (Fig. 2B). Similar results were observed when MRC-5 cells were infected with ROka12DR (a rescued version of ROka12D in which a full-length copy of ORF12 is inserted into the ORF12 deletion mutant ROka12D [21]) and treated with the PI3K inhibitor (Fig. 2C). While wild-type ROka and ROka12DR triggered higher levels of Akt phosphorylation than did ROka12D, addition of the PI3K inhibitor greatly reduced the levels of Akt activation by ROka and ROka12DR. These results suggest that the VZV ORF12 activation of Akt is dependent on PI3K and independent of ERK.

ORF12 protein associates with the p85 regulatory subunit of PI3K. Many viral proteins that activate the PI3K/Akt pathway either form a complex with the p85 regulatory subunit of PI3K (e.g., influenza A virus NS1 protein [24], bursal disease virus VP5 [25], HSV-1 VP11/12 [23]) or activate kinases upstream of Akt, such as Skp1 (myxoma virus M-T5 protein [26]) or Ras (EBV

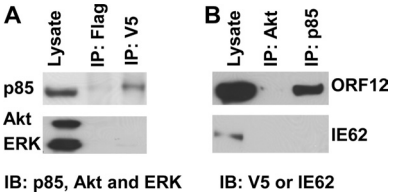

FIG 3 ORF12 protein associates with the p85 regulatory subunit of PI3K. MeWo cells were infected with ROka12DR (which expresses ORF12 protein with a V5 epitope tag [21]) for 36 h, and cell lysates were immunoprecipitated (IP) with antibody to p85, Akt, V5, or Flag and immunoblotted (IB) with antibody to p85, Akt, or ERK (A) or V5 or VZV IE62 protein (B).

LMP2A [11], HSV-2 ICP10PK [27]). Some viral proteins directly associate with Akt (e.g., NS1 of influenza A virus [28], HBx protein of hepatitis B virus [29], or M-T5 protein of myxoma virus [30]). Because the activation of Akt by ORF12 is dependent on PI3K, we reasoned that the VZV ORF12 protein might directly associate with PI3K regulatory subunits or Akt to affect Akt activity. To test this, MeWo cells were infected with ROka12DR, which expresses ORF12 protein with a V5 tag for 36 h, infected cell lysates were prepared, and immunoprecipitations were performed using antibodies to V5 (which was fused to ORF12 protein), p85, Flag, or Akt. The immune complexes were separated by SDS-PAGE and immunoblotted with antibodies to p85 or V5 (to detect ORF12 protein). p85 protein was detected by immunoprecipitation with antibody to V5 but not Flag (Fig. 3A). Conversely, ORF12 protein was detected with antibody to p85 but not Akt (Fig. 3B). As a control, Akt and ERK were not coimmunoprecipitated with antibody to V5 (Fig. 3A), and VZV IE62 was not coimmunoprecipitated with antibody to p85 (Fig. 3B).

ORF12 protein triggers phosphorylation of GSK-3β. Akt regulates downstream effectors such as mTOR1 and GSK-3 for protein synthesis, cell cycle regulation, and glucose metabolism. GSK-3 is a multifunctional serine/threonine kinase. Several known GSK-3 substrates participate in a wide spectrum of cellular processes, including glycogen metabolism, transcription, translation, cytoskeletal regulation, intracellular vesicular transport, cell cycle progression, and apoptosis (31). Two isoforms of GSK-3, GSK-3α (51 kDa) and GSK-3β (47 kDa), have been reported in mammals. GSK-3β is constitutively active in resting cells, and the treatment of cells with insulin or growth factors inactivates GSK-3 through a PI3K-dependent mechanism. PI3K-induced activation of Akt results in the phosphorylation of GSK-3β, which inhibits GSK-3 activity. Because VZV infection increases the level of phosphorylation of GSK-3β (20), and ORF12 protein activates Akt, we sought to determine whether ORF12 protein triggers the phosphorylation of GSK-3β in MRC-5 cells. While infection of MRC-5 cells with VZV ROka induced phosphorylation of GSK-3β, infection of cells with ROka12D resulted in less phosphorylation of GSK-3β at all time points than did infection of cells with ROka (Fig. 4A). Phosphorylation of mTOR at serine 2449, another signaling molecule downstream of the Akt pathway, was generally not affected by VZV ROka (Fig. 4A).

In addition to GSK-3β and mTOR, which are downstream targets of Akt, we also studied the effect of VZV ORF12 protein on upstream targets of Akt, including PTEN, Rictor (a component of the mTOR2 complex), and PI3K subunits p85 and PI3K III (Fig. 4A), which may affect Akt activity. While the levels of full-length p-PTEN did not change with VZV ROka infection, levels of

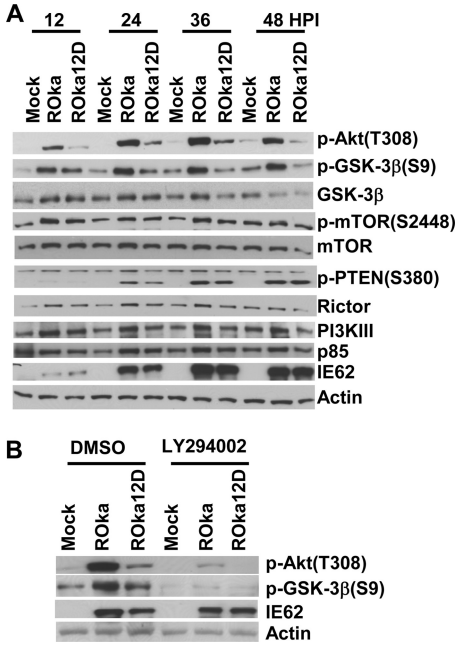

FIG 4 ORF12 protein triggers phosphorylation of GSK-3β. (A) MRC-5 cells were infected with ROka or ROka12D at 0.1 PFU/cell, and cell lysates were collected at the indicated times after infection and immunoblotted with various antibodies. (B) MRC-5 cells were infected with ROka or ROka12D at 0.1 PFU/cell for 24 h and treated with DMSO or 20 μM LY294002 for an additional 6 h, and cell lysates were immunoblotted with antibodies to p-Akt, p-GSK-3β, VZV IE62, and actin.

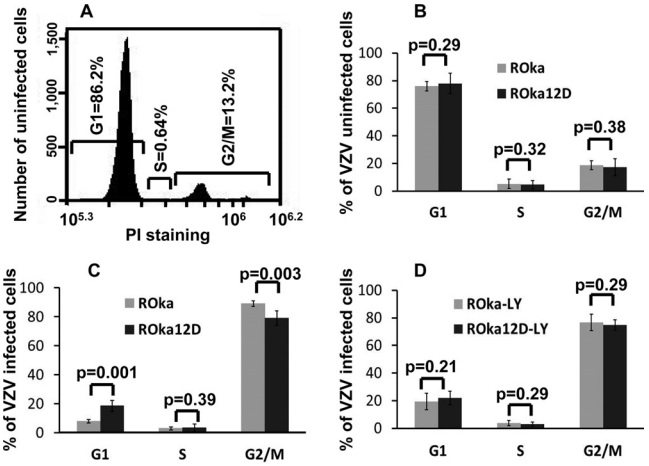

FIG 5 ORF12 protein regulates cell cycle progression. (A) Flow cytometry of MRC-5 cells stained with propidium iodide (PI) shows the percentage of cells in the G_1, S, or G_2/M phase of the cell cycle. Confluent MRC-5 cells were infected with MeWo cell-associated virus at 0.1 PFU/cell for 24 h, stained with PI and antibody to VZV glycoprotein E (gE), and gated for gE-negative (uninfected) cells (B) or gE-positive (VZV-infected) cells (C). (D) Confluent MRC-5 cells were treated with 10 μM LY294002 (LY) for 2 h before infection with ROka or ROka12D at 0.1 PFU/cell, stained with PI and antibody to VZV gE, and gated for gE-positive (VZV-infected) cells. Data were collected from 5 independent experiments using an Accuri C6 flow cytometer (BD Biosciences). *P* values were obtained with a one-tailed *t* test.

the short isoform of p-PTEN increased similarly in cells infected with VZV ROka or with ROka12D. In contrast, infection of cells with VZV ROka or ROka12D generally did not affect the levels of Rictor, PI3K III, or p85.

To determine whether the phosphorylation of GSK-3β is dependent on Akt, MRC-5 cells were infected with ROka or ROka12D for 24 h, the PI3K inhibitor LY294002 was added to inhibit Akt activity, and 6 h later cell lysates were prepared. Both ROka- and ROka12D-infected cells showed much less phosphorylation of GSK-3β in the presence of the PI3K inhibitor (Fig. 4B), indicating that VZV activation of GSK-3β is partially dependent on activation of the PI3K/Akt pathway.

ORF12 protein activation of PI3K/Akt regulates cell cycle procession. Since ORF12 protein activates Akt and GSK-3β, which are both important for cell cycle progression (32), and a prior study reported that VZV alters cell cycle progression by reducing the G_1 cell population and increases the S and M phase cell populations (33), we postulated that ORF12 protein may affect cell cycle progression. Confluent monolayers of MRC-5 cells were infected with ROka or ROka12D, and at 24 h after infection, cells were treated with trypsin, fixed with ethanol, stained with PI and VZV gE, and analyzed by flow cytometry to measure cell DNA content in VZV-infected (VZV gE-positive), and uninfected (gE-negative) cells. Cells were determined to be in the G_1, S, or G_2/M phase based on PI staining, and the percentage of cells in each phase of the cycle was calculated (Fig. 5A). Consistent with previous results (33), infection with VZV ROka at 24 h triggered virus-infected cells to progress from the G_1 phase into M phase, as shown by a reduction in the number of cells in the G_1 phase and an increase in the numbers of cells in the M phase in VZV-infected (VZV gE-positive) cells (Fig. 5C) compared to those in the uninfected (VZV gE-negative) cells (Fig. 5B). Using the mean values of 5 experiments, the percentages of cells in the G_1 phase fell from 75.9% in uninfected cells to 7.9% in VZV ROka-infected cells, the percentages of cells in M phase increased from 18.8% in uninfected cells to 89.1% in ROka-infected cells, and the percentages of cells in S phase decreased from 5.3% in uninfected cells to 3.0% in ROka-infected cells. While infection with VZV ROka12D also triggered infected cells to progress from the G_1 phase to the M phase of the cell cycle, the effect was significantly less than that for infection with ROka. The percentage of cells in the G_1 phase was 18.5% with ROka12D compared to 7.9% with ROka ($P < 0.001$), the percentage in M phase was 79.1% with ROka12D compared to 89.1% with ROka ($P < 0.003$), and the percentage in S phase was 3.4% with ROka12D compared to 3.0% with ROka ($P < 0.39$). These results indicate that ORF12 protein is important for the progression of virus-infected cells through the cell cycle; however, since the effect of ORF12 was only partial, other viral proteins probably contribute to VZV cell cycle regulation.

To determine if PI3K is important for the effect of ORF12 protein on the cell cycle, MRC-5 cells were treated with the PI3K inhibitor LY294002 for 2 h and then infected with ROka or ROka12D at 0.1 PFU/cell (Fig. 5D). The concentration of LY294002 used (less than that used in the experiment shown in Fig. 2B) did not adversely affect VZV replication. Inhibition of PI3K activity had little effect on the cell cycle progression of uninfected MRC-5 cells (data not shown), but it reduced the difference between the percentages of ROka- and ROka12D-infected cells in the G_1 and M phases (Fig. 5D). These results indicate that the effect of VZV ORF12 protein on cell cycle progression is dependent on PI3K.

VZV ORF12 protein directly increases levels of cyclin B1 in MRC-5 cells. The regulation of cell cycle progression is a complex

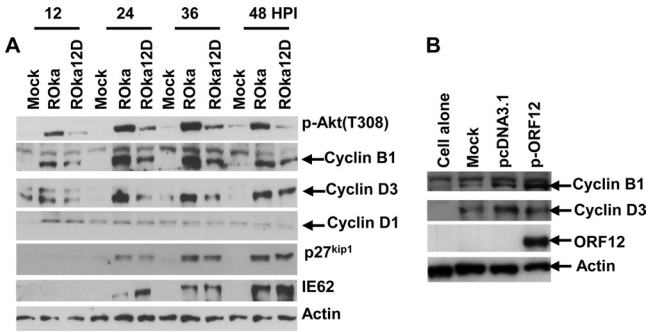

FIG 6 ORF12 protein directly upregulates expression of cyclin B1. (A) Deletion of ORF12 from VZV reduces the level of cyclin B1 and cyclin D3 in virus-infected cells. MRC-5 cells were infected with ROka or ROka12D at 0.1 PFU/cell, cell lysates were collected at the indicated times after infection, and immunoblotting was performed with various antibodies. (B) MRC-5 cells were transfected with pcDNA3.1, p-ORF12, or no plasmid (mock) or left untransfected (cells alone); after 72 h, cell lysates were prepared, and immunoblotting was performed with various antibodies.

process involving many cellular factors, including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors. A prior study reported that VZV infection alters cell cycle progression, resulting in increased levels of cyclins B1 and D3 and CDK inhibitor $p27^{Kip1}$ during VZV infection (33). Since ORF12 protein activates Akt and regulates cell cycle progression, and Akt is required for the regulation of cyclin D and CDK activity for early mitosis (3), we postulated that ORF12 protein might be partially responsible for the increase in cyclins B1 and D3 and/or $p27^{Kip1}$ (a CDK inhibitor) during VZV infection. Confluent MRC-5 cells were infected with ROka or ROka12D for various times, and immunoblotting was performed for detection of p-Akt (T308), cyclin B1, cyclin D1, cyclin D3, and $p27^{Kip1}$. The expression of cyclin B1 and cyclin D3 was induced in cells infected with VZV ROka and peaked at 24 to 36 h after infection (Fig. 6). In contrast, cells infected with ROka12D showed less induction of cyclin B1 and cyclin D3 expression at all time points compared to that of ROka-infected cells. While the levels of cyclin D1 and $p27^{Kip1}$ were increased at 12 h and 24 h after infection with VZV, respectively, there were no differences in the cells infected with ROka or ROka12D. The expression of ORF12 alone in MRC-5 cells upregulated cyclin B1 (Fig. 6B) but not cyclin D3, suggesting that the effect of ORF12 on cyclin D3 is indirect. These results indicate that VZV ORF12 directly increases the level of cyclin B1, which may help to regulate cell cycle progression.

DISCUSSION

VZV infection activates cellular signaling pathways such as ERK, p38, JNK, and PI3K/Akt for optimal viral replication (20, 33–35). Previously, we reported that the VZV ORF12 tegument protein is responsible for the activation of ERK1/2 and p38 (21). Here, we demonstrate that VZV ORF12 protein also activates the PI3K/Akt/GSK-3β pathway and increases the levels of cyclin B1 and cyclin D3 to regulate cell cycle progression.

The PI3K/Akt pathway is a critical pathway for cell proliferation, cell survival, protein synthesis, and glucose metabolism. HSV encodes three genes which have been reported to activate Akt, and a fourth protein that functions as an Akt surrogate. HSV VP11/12, the homolog of VZV ORF12 protein, was recently reported to activate PI3K/Akt in fibroblasts and T cells (23). HSV ICP10 phosphorylates Akt and protects neuronal cells from apoptosis (6, 36). HSV latency-associated transcript (LAT) is associated with the phosphorylation of Akt in neuroblastoma cells (37). Finally, the HSV US3 protein functions as an activated form of Akt to phosphorylate several proteins in human dermal fibroblasts (38, 39). Both VZV and HSV establish latent infections in neurons, and PI3K/Akt has been shown to be important for HSV to establish a latent infection (40). The Akt pathway is also important for HSV entry, including the transport of incoming HSV to the nuclear periphery (27, 41). Thus, Akt activation is important for human alphaherpesviruses and might have roles in VZV entry, establishment of latency, and protection of VZV-infected cells from apoptosis.

We found that activation of Akt by the VZV ORF12 protein was dependent on PI3K. While Rahaus and colleagues reported that VZV also increased the phosphorylation of Akt, they also found that the activation of Akt was not dependent on PI3K (20). We used MRC-5 human diploid fibroblasts for most of our experiments, while Rahaus and colleagues used only melanoma cells. We found that the effects of VZV on signaling proteins were more prominent in MRC-5 cells than in melanoma cells. The latter are transformed cells, and the PI3K/Akt pathway is known to be constitutively activated in these cells, such that PI3K/Akt is a target for chemotherapy for melanoma (42, 43). Thus, some of the differences between our findings and those of Rahaus and colleagues may be due to the cell lines used.

VZV ORF12 protein triggered phosphorylation of GSK-3β, which was dependent on Akt activity. Activation of the EBV receptor, gp350, on the surface of B cells induces phosphorylation of GSK, which is dependent on PI3K (44). HSV also induces phosphorylation of GSK-3α/β but through the US3 protein kinase (38). While HSV VP11/12 and VZV ORF12 protein both activate PI3K/Akt, HSV VP11/12 does not activate GSK-3β (23). In addition, while VZV ORF12 protein phosphorylates ERK and Akt, HSV VP11/12 only activates Akt. VZV ORF12 protein activates ERK, p38, and Akt, while HSV ICP10 phosphorylates ERK and Akt but not p38 (45). Thus, while VZV ORF12 protein and HSV VP11/12 share many activities, the viral proteins have distinct properties as well.

Our findings show that VZV ORF12 protein activates Akt to trigger GSK-3β phosphorylation and increase the levels of cyclin B1 and cyclin D3, which may regulate cell cycle progression. Activation of the cell cycle with a corresponding increase in intracellular nucleotides for cell division would be advantageous for viral replication. VZV replicates efficiently in a variety of cell types, including neurons which do not divide and are in the G_0 phase of the cell cycle, peripheral T cells and dermal fibroblasts that are usually in the G_1 phase of the cell cycle and are not dividing, human diploid fibroblasts that divide unless contact inhibited, and melanoma cells that rapidly divide. Activation of the cell cycle by VZV could drive nondividing or quiescent cells from the G_0 or G_1 phase into S phase and provide of pool of nucleotides for virus replication. We found that VZV ORF12 protein contributes to the progression of virus-infected cells from the G_1 phase into the M phase of the cell cycle and that this effect is dependent on PI3K. The effect of ORF12 on the cell cycle was somewhat modest, indicating that other viral proteins likely contribute to VZV cell cycle regulation.

The VZV ORF12 protein increased the levels of cyclin B1 and cyclin D3 in virus-infected cells. Cyclin B1 binds to and activates

CDK1, which phosphorylates lamin in the nucleus and contributes to breakdown of the nuclear membrane (46), although this has not been shown to occur in VZV-infected cells. Cyclin B1 promotes the progression of cells into the G_2-M checkpoint. Cyclin D3 moves cells from the G_1 phase of the cell cycle into the S phase by activating CDK4/6. The increase in levels of cyclin B1 and cyclin D3 with VZV ORF12 protein was associated with reduced numbers of cells in the G_1 phase and increased numbers of cells in the G_2/M phase. This differs from HSV infection, in which the virus blocks the progression of cells from the G_1 phase into S phase through the viral proteins ICP0 (47), ICP27 (48), and US1/US1.5 (49). Dysregulation of cell cycle progression by VZV is postulated to be controlled by transcription factors that are regulated by mitogen-activated protein kinases (50). We found that regulation of the cell cycle by VZV ORF12 protein was dependent on PI3K, which supports this hypothesis. This is further bolstered by observations that the inhibition of intracellular signaling pathways (34, 35) or cell cycle regulators (51, 52) by small molecules reduces VZV replication.

In summary, we have found that VZV ORF12 protein activates the PI3K/Akt pathway and that some, but not all, of the known downstream targets of PI3K/Akt are upregulated. ORF12 protein triggered the phosphorylation of GSK-3β but not mTOR and increased the levels of cyclin B1 and cyclin D3 but not cyclin D1. ORF12 protein regulation of cell cycle progression, from the G_1 phase to the M phase, was dependent on PI3K.

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